APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2007, p. 2661–2672 Vol. 73, No. 8
0099-2240/07/$08.00+0 doi:10.1128/AEM.00005-07

Comparative High-Density Microarray Analysis of Gene Expression during Growth of *Lactobacillus helveticus* in Milk versus Rich Culture Medium▽

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Received 2 January 2007/Accepted 4 January 2007

Lactobacillus helveticus **CNRZ32 is used by the dairy industry to modulate cheese flavor. The compilation of a draft genome sequence for this strain allowed us to identify and completely sequence 168 genes potentially important for the growth of this organism in milk or for cheese flavor development. The primary aim of this study was to investigate the expression of these genes during growth in milk and MRS medium by using microarrays. Oligonucleotide probes against each of the completely sequenced genes were compiled on maskless photolithography-based DNA microarrays. Additionally, the entire draft genome sequence was used to produce tiled microarrays in which noninterrupted sequence contigs were covered by consecutive 24-mer probes and associated mismatch probe sets. Total RNA isolated from cells grown in skim milk or in MRS to mid-log phase was used as a template to synthesize cDNA, followed by Cy3 labeling and hybridization. An analysis of data from annotated gene probes identified 42 genes that were upregulated during the growth of CNRZ32 in milk ($P < 0.05$), and 25 of these genes showed upregulation after applying Bonferroni's adjustment. The tiled microarrays identified numerous additional genes that were upregulated in milk versus MRS. Collectively, array data showed the growth of CNRZ32 in milk-induced genes encoding cell-envelope proteinases, oligopeptide transporters, and endopeptidases as well as enzymes for lactose and cysteine pathways, de novo synthesis, and/or salvage pathways for purines and pyrimidines and other functions. Genes for a hypothetical phosphoserine utilization pathway were also differentially expressed. Preliminary experiments indicate that cheese-derived, phosphoserine-containing peptides increase growth rates of CNRZ32 in a chemically defined medium. These results suggest that phosphoserine is used as an energy source during the growth of** ***L. helveticus*** **CNRZ32.**

Lactobacillus helveticus CNRZ32 (1) is a gram-positive, nonspore-forming, catalase-negative, microanaerophilic rod (20) with a G+C content of 37.1% (22). Representatives of this species are obligately homofermentative, thermophilic, lactic acid bacteria (LAB) that are used as starter cultures in the manufacturing of a variety of fermented dairy products, such as yogurt and mozzarella and Swiss cheeses (14), and as flavor adjunct cultures in other types of cheese, such as Gouda (1). The use of *L. helveticus* in the production of dairy foods has received increased attention because of this organism's ability to generate peptides with antihypertensive (35) and immunomodulating (24) properties from casein during milk fermentation. Like all lactobacilli, *L. helveticus* is a fastidious organism, requiring exogenous supplies of specific carbon and nitrogen sources, nucleotides, vitamins, and minerals for growth (20). Since *L. helveticus* CNRZ32 has multiple amino acid auxotrophies, its rapid growth in milk relies on a complex proteolytic enzyme system to obtain essential amino acids from caseins and other milk proteins (5, 6).

Lactose is the primary carbon source for microbial growth in milk, so the growth of *L. helveticus* in milk requires an enzymatic system to utilize this carbohydrate (11). Other potential sources of energy for microbial growth in milk and cheese include citrate (17), trace carbohydrates (such as ribose and *N*-acetylglucosamine) derived from milk glycoproteins and glycolipids (13, 48), nucleotides, nucleosides, and their precursors, formic and orotic acids (39, 40, 41).

Casein-derived peptides formed during the ripening of cheese are rich in phosphoserine (serP) residues (28). The composition of phosphopeptides varies considerably in different cheeses, and while some cheeses accumulate free serP at high levels during maturation, only trace amounts of this compound are registered in other varieties (10). The variation in serP accumulation suggests that serP, either in the form of a free amino acid or as a part of a phosphopeptide, can be utilized by cheese microorganisms, including *L. helveticus*, and may serve as an additional source of metabolic energy. Indeed, the draft CNRZ32 genomic sequence contains a gene cluster that may encode a pathway for serP utilization.

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▽ Published ahead of print on 23 February 2007.

When milk is fermented into cheese, *L. helveticus* and other LAB contribute to cheese flavor development during the ripening period through several basic mechanisms that include carbohydrate fermentation, conversion of milk proteins into peptides and free amino acids, catabolism of amino acids into aroma compounds, hydrolysis of milk lipids into free fatty acids, followed by their conversion to esters, and citrate catabolism (12). Our group has compiled a draft (fourfold random coverage) sequence for the 2.4-Mb genome of *L. helveticus* CNRZ32 (37). The use of this information for functional genomic studies would allow us to better understand the molecular events occurring during cheese ripening and, ultimately, to improve flavor development in bacteria-ripened cheeses.

For this study, we screened the draft sequence for genes that might be important for the growth of *L. helveticus* CNRZ32 in milk and/or for cheese flavor development. A total of 168 of such genes were identified whose products are expected to be involved in proteolysis, citrate utilization, metabolism of lipids and amino acids, carbohydrate utilization, and other functions. The primary aim of this study was to investigate changes in expression among this subset of genes during the growth of *L. helveticus* CNRZ32 in milk versus laboratory medium using high-density DNA microarrays. Additionally, noninterrupted sequence fragments ("contigs") were employed to generate a "tiled" microarray by depositing consecutive probes designed to represent all of the available sequence information.

The experiments allowed the identification of a number of genes that are expressed differentially during the growth of *L. helveticus* CNRZ32 in milk. In particular, the results suggested an ability of this organism to generate metabolic energy from phosphoserine residues in milk caseins and to convert citrate into succinate, an important flavor compound. These data provide new insight to *L. helveticus* physiology and identify targets for future functional genetics experiments in this species.

MATERIALS AND METHODS

Bacteria and culture media. *L. helveticus* CNRZ32 (1) was maintained in a laboratory collection as a glycerol stock at −80°C and propagated at 42°C in MRS broth (Difco Laboratories, Detroit, MI). Skim milk for these experiments (Babcock Dairy, Madison, WI) was double steamed for 20 min with a 2-h, 42°C incubation between treatments. Chemically defined medium (CDM) base was prepared as described by Christensen and Steele (6) and contained a limiting concentration of glucose (3 mM); pH was adjusted to 6.0 with NaOH. Potential growth promoters, namely serine, phosphoserine (both from Sigma), and phosphopeptides isolated from Herrgard cheese ("pool 3" [28]), were filter sterilized before the addition to autoclaved CDM. Cells for growth experiments were propagated from frozen stocks by passage through two sequential subcultures in MRS broth at 42°C for 17 h. The cells were washed in saline and inoculated to a final calculated optical density at 600 nm (OD_{600}) of 0.02 or 0.005 in 400 ml of milk or MRS broth, respectively. When CDM was used as the growth medium, the cells were inoculated at an OD_{600} of 0.05 into 5 ml of medium. Milk and MRS cultures were incubated at 42°C until cells reached mid-log phase (approximately 9 h, as determined by preliminary trials), while CDM cultures were grown for 24 h. The final pH values of MRS and milk cultures were 5.5 to 5.6. Because a comparison of gene expression profiles between the cultures of different growth phases was not the subject of this study, this point of the growth curve was chosen arbitrarily. Three independent growth experiments in milk and MRS were performed, and two identical samples were taken from each culture and used for the RNA isolation. A total of six RNA samples for each growth condition were used to independently produce labeled cDNA for microarray experiments. Growth experiments in CDM were performed in triplicate. The growth rate was defined as the maximum slope of log OD_{600} versus time graphs.

RNA isolation and purification. Cultures were mixed by vortexing with two volumes of RNAprotect reagent (QIAGEN, Inc., Valencia, CA) containing 100 μg/ml of rifampin (Sigma, St. Louis, MO) and incubated for 5 min at room temperature, followed by centrifugation at 5,500 × *g* for 15 min at 4°C. The cell pellets were resuspended in 5 ml of lysozyme solution (20 mg/ml) containing 0.1 mg/ml of rifampin and incubated for 25 min at 37°C. After repeated centrifugation, the pellets were resuspended by vortexing in 5 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and incubated for 10 min at room temperature. Next, 1 ml of chloroform was added, followed by vortexing for 15 s and incubation for 10 min at room temperature. The mixtures were centrifuged at 16,000 × *g* for 20 min at 4°C, 2.5 ml of the upper aqueous phase was transferred into a fresh tube, mixed with 2.5 ml of isopropanol, and incubated at room temperature for 10 min and RNA was pelleted at 16,000 × *g* for 20 min at 4°C. After washing in 75% ethanol, the RNA pellet was dried and dissolved in 100 μl of water. The isolated total RNA was treated with 5 U of RQ1 DNase I (Promega, Madison, WI) and then purified using the RNeasy purification system (QIAGEN).

cDNA synthesis and labeling. cDNA was synthesized from 12 μg of total RNA using random hexamer primers (Amersham Bioscience, Piscataway, NJ) and SuperScript II reverse transcriptase (Invitrogen). After synthesis, template RNA was digested with RNase H (Promega) and RNase A (Epicenter, Madison, WI), and cDNA was purified using the QIAquick PCR purification kit (QIAGEN) and then fragmented into approximately 70-base fragments using appropriately diluted RQ1 DNase I (Promega). Fragmentation efficiency was determined with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Fragmented cDNA was end labeled with biotin-N6-ddATP (NEN/Perkin-Elmer, Boston, MA) using terminal deoxynucleotidyl transferase (Promega), followed by the concentration of labeled sample on Microcon YM-10 columns (Millipore, Bedford, MA).

Microarray design and production. Based on the sequence and annotation data for *L. helveticus* CNRZ32, high-density, photolithography-based, monoplex (one array per chip) microarrays were designed and produced by NimbleGen Systems, Inc. (Madison, WI). The arrays consisted of 24-mer probe pairs, 18 "perfect match" probes that were identical to the original sequence, and 18 "mismatch" probes that differed from the original sequence at the two center positions for each of 168 polished genes. Genes selected included those whose products are expected to be involved in proteolysis/milk protein utilization/oligopeptide transport (58 genes), citrate utilization (13 genes), lipid and amino acid metabolism (9 and 27 genes, respectively), carbohydrate utilization (9 genes), and 50 other genes predicted to be involved in exopolysaccharide synthesis, nucleotide metabolism, solute transport, competence, or cell division processes. Additionally, "tiled" probe pairs were designed to cover the entire draft sequence in both the "sense" and "antisense" (reverse-complement) directions, with an average distance of approximately 30 nucleotides between probes. Each of the probe pairs was synthesized in a computer-generated randomized pattern on the array.

Hybridization, Cy3 conjugation, and antibody amplification. All hybridization, staining, and processing of arrays were performed by personnel at NimbleGen Systems essentially as previously described by Ulijasz et al. (44). In brief, hybridizations were carried out at 45°C for 16 h on a rotisserie-like apparatus (HybriWheel) to enhance uniformity of hybridizations across the array surface. After hybridization, the arrays were washed in buffers of various levels of stringency and then streptavidin was conjugated to the end-labeled biotin, followed by biotin-anti-streptavidin in the presence of normal goat immunoglobulin G, and finally conjugated to a Cy3 streptavidin.

Array scanning, data extraction, and analysis. Arrays were scanned using an Axon model 4000 scanner (Molecular Devices Corporation, Union City, CA) and the data were extracted using NimbleScan software. Array normalization was performed using the quantile normalization method of Bolstad et al. (2). Normalized expression values for the individual probes were used to obtain the expression values for a given open reading frame (ORF) by using the robust multiarray average (RMA) procedure as previously described by Irizarry et al. (18). Finally, *n*-fold change ratios (*R*) were calculated using the RMA-processed expression values (RMA calls) obtained for a particular gene in milk and MRS cultures.

For the tiled arrays, a manual probe-by-probe scan was conducted to locate clusters of three or more consecutive probes which produced an average signal intensity that was at least 1.95-fold higher in milk-grown than in MRS-grown cells. Genome sequence regions that corresponded to these probes were then used in similarity searches with online BLASTx and BLASTp algorithms (http://www.ncbi.nlm.nih.gov/BLAST/) to identify potential ORF products. Finally,

TABLE 1. Oligonucleotides used for real-time quantitative PCR

Gene	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)	Annealing temp (°C)
asnA	AACTGTCCGTGATACTGAAGCA	CGAACATGGAGCAGACATTCT	104	59
cysE	TTCCTAAGTCAACATGCCGCC	CGCCATGCAGGATAGTGACG	148	58
dapA	CGCCTTAGTCGTTGTACCG	AATGGCATCTTAACGTTGTCG	93	59
serA	TACTCCGCTCATAACAGTGG	CAATAACGGCTAATGTCTTGC	103	59
L-ldh	ACCAAGAAGTTAAGGACATGGC	CCTTAGCGATCATTGCTGAAGC	93	62
clpP	GATGCACAAGACAACACTAAGG	TACCATTGCATAGTTGATACG	130	58
oppA	CAGTTGAACAATATGGCAAGAGC	AAGGTTCGAGCCTGTCCAACC	95	58
oppC	ACTTAGGTCGTTCACTTGGTCA	TCCATCCGGATACTATTCCGTA	123	59
pepO2	TGGCTTTCAACCTGCTCAAGCT	TCTTACGATCAGGTTCAACTTCG	137	61
pepT2	GCCGGTATGAGTATGTAACCG	AAGTGGACGTTCATCTCTGCC	113	62
prtH	TAATCCTAGCGAGCAACATGG	TAGCTAGTAATTGAGCTTCTGG	113	58
prtH2	TACCAAGCAGGTGGTAACGC	ACTGTGATCGCACTTCTGGC	89	57
purA	ACCGTGCTCATATCATCATGCC	GTTGGTCCAATACCGTTCTTGG	106	61
pyrR	CGTGATGATCGCCATGATGC	CCTGTGTAGATTACATCATCG	116	57

normalized data for the probes covering the newly discovered ORFs were subjected to the RMA procedure and the expression ratios were calculated.

A mixed-model analysis of variance approach (49) was used to determine which annotated or newly discovered *L. helveticus* CNRZ32 ORFs were differentially expressed in milk or MRS medium. This statistical method was applied to test the expression changes of all annotated genes, i.e., independently of the *n*-fold change ratios. However, only those newly discovered ORFs which retained change ratios (*n*-fold) above or equal to 1.95 after applying the RMA treatment were subjected to further statistical analysis. To account for multiple testing, a Bonferroni's adjustment to an overall significance level of 0.10 was used as the cutoff value (i.e., $\alpha = 0.10/n$, where "*n*" is the number of ORFs tested). Finally, the use of arbitrary a priori *P* value cutoffs in conjunction with Bonferroni's correction proved useful in the analysis of microarray data (9, 33), so data in this study was also examined at two *P* values, 0.01 and 0.05, which are commonly used in biological experiments as arbitrary significance cutoffs. Results for a particular gene are presented as an *n*-fold change of expression, along with its cognate *P* value and whether the change was significant after Bonferroni's correction.

RT-PCR. Primers for 14 different genes (Table 1) were designed with GeneWorks software (IntelliGenetics, Inc., Mountain View, CA) and compared against the *L. helveticus* CNRZ32 genome sequence using BioEdit 5.0.6 software (www.mbio.ncsu.edu/BioEdit/bioedit.html) to verify that each annealed to a single locus in the genome. Primer pairs were predicted to have annealing temperatures that ranged from 55 to 61°C and to produce amplicons that ranged from 89 to 148 bp in length. Template DNA from *L. helveticus* CNRZ32 was used to determine optimal reaction conditions in real-time PCR (RT-PCR) for each primer pair to ensure the absence of any nonspecific amplification, and the identity of PCR products was confirmed by sequencing. Reactions were performed in an Opticon II thermal cycler (MJ Research, Reno, NV). Each reaction consisted of 5 μl of a solution of either template DNA or cDNA or water in the negative controls, 5 μl of primer mix (1.2 μM of each primer), and 10 μl of SYBR green mix (MJ Research). Blanks contained 10 μl of SYBR green mix plus 10 μl of water. RT-PCR was performed using two concentrations of cDNA (2 ng/μl and 0.02 ng/μl) obtained from milk or MRS-grown *L. helveticus* CNRZ32 cultures as described above. Triplicate reactions were run in 96-well plates. Amplicon quantification in RT-PCRs was performed by comparison with gene-specific standard curves constructed from known concentrations of individually purified amplicons. The obtained amplicon copy numbers were log transformed and used in the calculation of the expression change (*n*-fold) for a particular gene.

Nucleotide sequence accession numbers. The nucleotide sequences of 168 completely sequenced genes have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html). The accession numbers are given in Tables 2, 3, and 4. Microarray data analyzed in this study have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) and were assigned series record GSE7005.

RESULTS

Microarray expression profiling. Normalization and analysis of array hybridization signals found 28 of 168 annotated genes were differentially expressed at a level of 1.45-fold or greater, and 15 of these genes were induced at least 1.95-fold when *L. helveticus* CNRZ32 was grown in milk compared to that in MRS broth (Table 2). Application of the most stringent multiple comparison correction, Bonferroni's adjustment with overall cutoff value of 0.1, indicated that 25 genes were significantly upregulated in milk-grown cells. These genes included one gene, *pepT*, whose expression changed less than 1.45-fold. However, Bonferroni's correction is considered to be the most conservative approach to reduce type I (false-positive) errors and, in turn, would increase the potential for type II (false-negative) errors (38, 47). Further analysis using 0.01 and 0.05 as arbitrary a priori *P* value cutoffs revealed the upregulation of 17 additional genes, which brings the total number of polished genes significantly upregulated during the growth in milk to 42.

The expression data obtained from the tiled microarray identified 79 additional ORFs that were significantly upregulated during the growth of *L. helveticus* CNRZ32 in milk. These included 45 ORFs whose products show significant similarity to previously characterized proteins of known or predicted function (Table 5), 11 ORFs which may encode proteins significantly similar to the GenBank entries that lack predicted function (i.e., "hypothetical" or "conserved hypothetical" proteins), and 23 potential ORFs (i.e., upregulated nucleotide sequences for which a valid RBS and start codon could be assigned) whose products did not have a significant match in the protein database (data not shown).

Based on the combined results from annotated and tiled microarrays, growth in milk led to the upregulation of 21 genes that encode enzymes from proteolytic systems (proteinases, peptidases, and oligopeptide transporters, presumably participating in casein degradation/assimilation), 4 genes involved in biosynthesis or metabolism of serine and cysteine, 5 genes from carbohydrate fermentation pathways, 27 genes related to nucleotide metabolism, 3 genes of citrate utilization pathways, 4 genes for components of three different ABC-type transport systems, and 4 genes that could encode proteins of other functions. Additionally, the tiled arrays revealed significant upregulation of 18 phage-related proteins during the growth of *L. helveticus* CNRZ32 in milk (Tables 2 and 5).

TABLE 2. Annotated genes induced during growth of *Lactobacillus helveticus* CNRZ32 in milk

Protein type or function	Gene	Protein encoded	GenBank accession no.	Induction[a]	*P* value[b]
Proteolytic enzyme system	*prtH*	Cell envelope-associated proteinase	AAD50643	5.81*	<0.0001
	prtH2	Cell envelope-associated proteinase	DQ826130	4.34	0.0005
	prtM	Proteinase activation protein precursor	DQ826131	3.00	0.0016
	oppA	Oligopeptide ABC transporter, substrate-binding component	DQ826119	1.52	0.0016
	oppB	Oligopeptide ABC transporter, permease component	DQ826120	1.60*	<0.0001
	oppC	Oligopeptide ABC transporter, permease component	DQ826120	1.62*	<0.0001
	oppD	Oligopeptide ABC transporter, ATP-binding protein	DQ826120	1.45*	<0.0001
	oppF	Oligopeptide ABC transporter, ATP-binding protein	DQ826120	1.39	0.0037
	pepE	Aminopeptidase E	U77050	1.22	0.0051
	pepN	Aminopeptidase N (lysyl/alanine aminopeptidase)	U08224	1.15	0.0205
	pepR	Prolyl aminopeptidase (prolinase)	U05214	1.15	0.0438
	pepT	Aminotripeptidase (peptidase T)	DQ826128	1.31*	0.0003
	pepT2	Aminotripeptidase (peptidase T2)	DQ826129	1.58*	0.0004
	pepO2	Endopeptidase	DQ826126	1.86*	0.0002
	pepO	Neutral endopeptidase (endopeptidase O)	AF019410	1.18	0.0210
	pepV	Xaa-His dipeptidase (carnosinase)	AF012085	1.31	0.0047
	pepX	Xaa-Pro dipeptidyl-aminopeptidase	U22900	1.13	0.0354
	htpX	Protease, heat shock protein homolog	DQ826108	1.12	0.0302
	ypwA/*amd1*	Carboxypeptidase/aminoacylase	DQ826137	1.78*	0.0004
Amino acid metabolism	*ATase2*/*nifS2*	Aminotransferase class V/cysteine desulfurase	DQ826154	1.19	0.0113
	serA	Phosphoglycerate dehydrogenase	DQ826155	2.01*	<0.0001
	serC	Phosphoserine aminotransferase	DQ826155	2.07*	<0.0001
Carbohydrate metabolism and glycolysis	*lacM*	Beta-galactosidase, small subunit	DQ826053	1.56*	<0.0001
	lacS	Lactose permease (lactose-proton symporter)	DQ826054	1.17	0.0081
Lipase-esterase genes	*lip* (*con hyp069A1*)	Triacylglycerol lipase	DQ826066	1.10	0.0115
Nucleotide metabolism	*guaA*	GMP synthase, glutamine hydrolyzing	DQ826084	3.18*	<0.0001
	guaB	Inosine-5-monophosphate dehydrogenase	DQ826085	1.48*	<0.0001
	nrdF	Ribonucleoside-diphosphate reductase (class I), β-subunit	DQ826087	1.67*	<0.0001
	purA	Adenylosuccinate synthetase	DQ826091	6.40*	<0.0001
	purB	Adenylosuccinate lyase	DQ826091	4.79*	<0.0001
	purD	Phosphoribosylamine-glycine ligase	DQ826092	4.34*	<0.0001
	purF	Phosphoribosylpyrophosphate amidotransferase	DQ826092	5.73*	<0.0001
	purH	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase (bifunctional)	DQ826092	4.50*	<0.0001
	purL	Phosphoribosylformylglycinamidine synthase, synthetase domain (component II)	DQ826092	5.35*	<0.0001
	purM	Phosphoribosylformylglycinamidine cyclo-ligase	DQ826092	4.93*	<0.0001
	purN	Phosphoribosylglycinamide formyltransferase	DQ826092	3.90*	<0.0001
	purQ	Phosphoribosylformylglycinamidine synthase, glutaminase domain (component I)	DQ826092	5.43*	<0.0001
Citrate utilization	*citI*	Citrate lyase regulator	DQ826058	1.50*	0.0002
	citG	Phosphoribosyl-dephospho-CoA transferase	DQ826057	1.38	0.0028
	yflS/*citT*	Di- and tricarboxylate/possible citrate transporter	DQ826064	1.49*	<0.0001
Binding and transport	*ABC3 ATP3*	ABC transporter, ATP-binding protein	DQ678932	1.81	0.0005
Miscellaneous	*pbpC1*	D-Ala-D-Ala carboxypeptidase (penicillin binding protein)	DQ826089	1.20	0.0181

[a] Fold change in normalized microarray signal intensity during growth in milk versus MRS. Given are average values calculated from three independent repeats performed in duplicate. Asterisks indicate significance after applying Bonferroni's correction at an overall level of 0.1.
[b] *P* values were calculated by the mixed-model method.

Results from an annotated array showed a total of 61 genes to be significantly upregulated during the growth of *L. helveticus* CNRZ32 in MRS compared to that in milk ($P < 0.05$), including 17 genes that were significantly upregulated after applying Bonferroni's correction (Table 2). These included 19 genes that encode enzymes from the proteolytic system, 16 genes involved in amino acid biosynthesis and metabolism, 5 genes needed for carbohydrate utilization, 4 lipase or esterase genes, 5 genes involved in citrate catabolism, components of three distinct ABC-type transporters, and 9 genes that could encode proteins of other functions. These differences found between gene expression patterns in *L. helveticus* CNRZ32 grown in milk as opposed to those in MRS medium are summarized in Table 6.

RT-PCR validation. RT-PCR experiments were performed for 14 target genes with the same cDNA preparations used in array hybridizations. In general, the RT-PCR data showed good agreement with the microarray results, and there was a positive correlation ($r = 0.76$) between the two methods (Fig. 1). However, divergent results between the two platforms were observed for three genes, *asnA* (asparagine synthase), *cysE* (serine *O*-acetyltransferase), and *pyrR* (pyrimidine operon regulator). Microarray data indicated that *asnA* was significantly repressed in milk-grown cells according to the microarray analysis (Table 3), but RT-PCR showed its upregulation ($R = 1.49$), though the effect was not significant ($P > 0.05$). The second gene, *cysE*, was in turn found to be upregulated in milk-grown cells by microarray analysis (Table 2), but no significant difference in expression was

TABLE 3. Annotated genes induced during growth of *L. helveticus* CNRZ32 in MRS broth

Protein type or function	Gene	Protein encoded	GenBank accession no.	Induction[a]	*P* value[b]
Proteolytic enzyme system	*clpC*	ATP-dependent protease, ATPase subunit	DQ826089	1.17	0.0096
	clpP	ATP-dependent protease, proteolytic subunit	DQ826100	1.73*	<0.0001
	clpQ	ATP-dependent protease, peptidase subunit	DQ826101	1.35	0.0016
	clpX	ATP-dependent protease, ATPase subunit	DQ826102	1.18	0.0096
	clpY	ATP-dependent protease, ATPase subunit	DQ826101	1.25	0.0004
	dtpA1/oppA1	Di-/tri-/oligopeptide transport system, binding component	DQ826104	1.19	0.0044
	dptT	Di-/tripeptide transporter	DQ826105	1.68	0.0009
	eep	Membrane-associated Zn-dependent protease	DQ826106	1.26*	0.0004
	ftsH	ATP-dependent Zn protease (cell division protein)	DQ826083	1.25	0.0005
	gcp	*O*-Sialoglycoprotein endopeptidase	DQ826107	1.08	0.0073
	htrA	Serine protease	DQ826109	7.87*	<0.0001
	pcp	Pyrrolidone-carboxylate peptidase	DQ826121	1.10	0.0248
	pepD	Cytosolic nonspecific dipeptidase	U34257	1.36	0.0012
	pepD2	Cytosolic nonspecific dipeptidase	DQ826122	1.21	0.0167
	pepD4	Cytosolic nonspecific dipeptidase	DQ826124	1.23	0.0176
	pepI	Proline iminopeptidase	DQ826125	1.19	0.0135
	pepQ2	Xaa-Pro dipeptidase (prolidase)	DQ826127	1.32	0.0024
	prtM2	Protease maturation protein precursor	DQ826132	1.46	0.0008
	sipT	Signal peptidase I	DQ826135	1.35	0.0005
Amino acid metabolism	*Ald-ketoRed1/ycsN*	Oxidoreductase/aldo-keto reductase	DQ826148	1.12	0.0306
	asnB	Asparagine synthase, glutamine hydrolyzing	DQ826145	1.70*	<0.0001
	aspC	Aspartate aminotransferase	DQ826142	1.55*	<0.0001
	asd	Aspartate-semialdehyde dehydrogenase	DQ826142	1.56	0.0004
	asnA	Aspartate-ammonia ligase	DQ826144	1.46	0.0012
	ATase1/nifS1	Aminotransferase, class V/cysteine desulfurase	DQ826153	1.54*	0.0001
	ATase3	Aspartate/tyrosine/aromatic aminotransferase (aminotransferase, class I and II)	DQ826149	1.31	0.0056
	dapA	Dihydrodipicolinate synthase/*N*-acetylneuraminate lyase	DQ826142	2.00*	<0.0001
	dapB	Dihydrodipicolinate reductase	DQ826142	1.86*	<0.0001
	dapD	Tetrahydrodipicolinate *N*-succinyltransferase	DQ826142	2.12*	<0.0001
	dapE	Succinyldiaminopimelate desuccinylase/amino acid amidohydrolase	DQ826142	2.15*	<0.0001
	dltE/ydfG/fabG	Short-chain dehydrogenase (reductase)	DQ826150	1.10	0.0141
	ldhD	D-Lactate dehydrogenase	U07604	1.09	0.0460
	ldhL	L-Lactate dehydrogenase	DQ826139	1.11	0.0321
	ykrU	Amidohydrolase	DQ826149	1.22	0.0425
Carbohydrate metabolism and glycolysis	*ccpA*	Transcriptional regulator/catabolite control protein	DQ826048	1.28	0.0006
	gapA	Glyceraldehyde-3-phosphate dehydrogenase	DQ826063	1.10	0.0116
	glcU	Glucose/ribose uptake protein	DQ826052	1.31	0.0012
	tpiA	Triosephosphate isomerase	DQ826055	1.25	0.0010
	ygaP/cggR	Transcriptional regulator/central glycolytic regulator	DQ826051	1.13	0.0098
Lipase-esterase genes	*cpd1*	2′,3′-Cyclic-nucleotide 2′-phosphodiesterase	DQ826067	1.86*	<0.0001
	glp1	Glycerophosphoryl diester phosphodiesterase	DQ826068	1.30*	0.0004
	hyp lip1	Predicted lipase/esterase	DQ826069	1.18	0.0052
	yhaO	Phosphoesterase	DQ826072	1.38	0.0007
Citrate utilization	*cilA* (*citF*)	Citrate lyase, α-subunit	DQ826056	1.49	0.0008
	cilB (*citE*)	Citrate lyase β-subunit	DQ826056	1.34*	0.0002
	citC	Citrate lyase ligase	DQ826056	1.17*	0.0002
	citD	Citrate lyase, γ-subunit	DQ826056	1.35	0.0012
	frdC3	Fumarate reductase, flavoprotein subunit	DQ826062	1.28	0.0057
Binding and transport	*ABC1 ATP1*	ABC transporter, ATP-binding protein	DQ826075	3.33*	<0.0001
	ABC1 MC1 (*sufD*)	ABC transporter involved in [Fe-S] cluster assembly, membrane/permease component	DQ826146	1.15	0.0042
	ABC2 ATP2	ABC transporter ATPase component	DQ826076	1.33	0.0136
	ABC4 ATP4	ABC transporter ATPase component	DQ826077	1.32	0.0085
	adh1	CoA-linked acetaldehyde dehydrogenase/iron-dependent alcohol dehydrogenase	DQ826152	4.31*	<0.0001
	comEA	Late competence protein	DQ826079	1.18	0.0395
	dacA	D-Ala-D-Ala carboxypeptidase/penicillin-binding protein 5/6	DQ826080	1.20	0.0102
	epsD	Exopolysaccharide biosynthesis protein (tyrosine-protein phosphatase)	DQ826082	1.10	0.0300
	groEL	Chaperonin	DQ826073	1.31	0.0016
	groES	Cochaperonin			
	pbpA	Membrane transpeptidase-transglycosylase/penicillin-binding protein	DQ826088	1.65*	<0.0001
	pbpC2	D-Ala-D-Ala carboxypeptidase/penicillin-binding protein	DQ826074	1.38	0.0077
	recA	Recombination protein	DQ826134	1.56	0.0007

[a] Fold change in normalized microarray signal intensity during growth in MRS versus milk. Given are average values calculated from three independent repeats performed in duplicate. Asterisks indicate significance after applying Bonferroni's correction at an overall level of 0.1.

[b] *P* values were calculated by the mixed-model method.

TABLE 4. Annotated genes which were not differentially expressed during growth of *L. helveticus* CNRZ32 in milk or MRS

Protein type or function	Gene	Protein encoded	GenBank accession no.
Proteolytic enzyme system	*clpE*	ATP-dependent protease ATP binding subunit	DQ826098
	clpE2	ATP-dependent protease ATP binding subunit	DQ826099
	dtpA	Di-/tri-/oligopeptide transport system, binding component	DQ826103
	hyp pep1	Peptidase, serine beta-lactamase-like superfamily	DQ826110
	hyp prt1	Membrane protease	DQ826111
	hyp prt2	Zn-dependent peptidase M16 family	DQ826112
	hyp prt3	Serine protease, PDZ family	DQ826113
	hyp prt4	Serine protease	DQ826114
	lepA	Leader peptidase	DQ826115
	lspA	Prolipoprotein signal peptidase	DQ826116
	map	Methionine aminopeptidase	DQ826118
	pepC	Aminopeptidase	Z30340
	pepD3	Dipeptidase	DQ826123
	pepE2	Endopeptidase	AAQ72431
	pepF	Endopeptidase	AY365129
	pepO3	Endopeptidase	AY365128
	pepQ	Xaa-Pro dipeptidase (prolidase)	AF012084
	radA	ATP-dependent serine protease	DQ826133
	srtA	Sortase (surface transpeptidase)	DQ826115
	ybnA	GTP-binding protein, HSR1-related	DQ826136
	ydiC	Glycoprotein endopeptidase	DQ826117
	ysdC	Aminopeptidase	DQ826138
Amino acid metabolism	*ans*	Asparaginase	DQ826141
	bcaT	Branched-chain amino acid aminotransferase	DQ826143
	cbl	Cystathionine β-lyase	DQ826147
	csd	Cysteine desulfurase	DQ826146
	cysE	Serine *O*-acetyltransferase	DQ826147
	cysK	Cysteine synthase	DQ826147
	glmS1	Glutamine-fructose-6-phosphate transaminase	DQ826151
	L-ldh2	L-Lactate dehydrogenase	DQ826140
Carbohydrate metabolism and glycolysis	*deoR1*	Transcriptional regulator	DQ826049
	fbaA	Fructose-1,6-biphosphate aldolase	DQ826050
Citrate utilization	*citR*	Translational regulator	DQ826056
	CitX	Triphosphoribosyl-dephospho-CoA synthetase	DQ826059
	frdC	Fumarate reductase, flavoprotein subunit	DQ826060
	frdC2	Fumarate reductase, flavoprotein subunit	DQ826061
	gabD	Succinic semialdehyde dehydrogenase	DQ826063
Lipase-esterase genes	*Cls*	Phosphatidylserine/cardiolipin synthase	DQ826065
	estA	Arylesterase	AF136284
	hyp lipA	Esterase/lipase	DQ826070
	hyp lipB	Esterase/lipase	DQ826071
Binding and transport	*ABC5 ATP5*	ABC transporter, ATP-binding protein	DQ826146
	ABC5 MC5	ABC transporter, membrane protein	DQ826146
Nucleotide metabolism	*pyrAA*	Carbamoyl-phosphate synthase, small subunit	DQ826093
	pyrAB	Carbamoyl-phosphate synthase, large subunit	DQ826093
	pyrB	Aspartate carbamoyltransferase	DQ826093
	pyrC	Dihydroorotase	DQ826093
	pyrR	Uracil phosphoribosyltransferase	DQ826093
Miscellaneous	*comC*	Type IV prepilin peptidase	DQ826078
	comEC	Late competence protein 3/DNA uptake protein	DQ826079
	dnaE	DNA polymerase III	DQ826081
	epsA	Transcriptional regulator of *eps* operon	DQ826082
	epsB	Exopolysaccharide biosynthesis protein (regulator)	DQ826082
	epsC	Exopolysaccharide biosynthesis protein (tyrosine-protein kinase)	DQ826082
	epsE	Exopolysaccharide biosynthesis protein (phospho-glucosyltransferase)	DQ826082
	intron	Group II intron protein	EF159953
	mdp	Mevalonate diphosphate decarboxylase	DQ826086
	mvk	Mevalonate kinase	DQ826086
	nifU	Nitrogen fixation protein homolog involved in [Fe-S] cluster formation	DQ826146
	pbpX	Penicillin binding protein	DQ826096
	ponA	Penicillin binding protein 1A	DQ826090
	thiJ	4-Methyl-5(β-hydroxyethyl)-thiazole phosphate biosynthesis protein	DQ826094
	uspA	Universal stress protein	DQ826095
	wzy	Polysaccharide polymerase	DQ826082
	wchF	Rhamnosyl transferase	DQ826082

recorded by RT-PCR ($R = 0.92$; $P > 0.05$). The third gene, *pyrR*, was not differentially expressed as judged by microarray analysis ($R = 0.90$; $P > 0.05$) but was upregulated based on the RT-PCR data ($R = 3.52$; $P < 0.01$). Therefore, no statistically significant opposite trends (i.e., upregulation instead of downregulation) were observed when the expression level of a particular gene was analyzed by two alternative methods. The slope of the regression line was found to be 2.48, which indicates that RT-PCR generally

TABLE 5. Putative genes (identified from the tiled microarrays) significantly induced during growth of *Lactobacillus helveticus* CNRZ32 in milk

Protein type or function	Gene	Protein encoded	Sequence ID/ contig name[a]	Probe position[b]	Top BLAST hit	Induction[c]	*P* value[d]
Proteolytic enzyme system	*prtH3*	Cell envelope-associated proteinase	178/002-6	6080-11055	CAD43138	3.58*	0.0002
	prtH5	Cell envelope-associated proteinase	1590/26611_R	1-4017	CAD43138	5.26*	<0.0001
Amino acid metabolism	*hyp1677-2*	Membrane serine/threonine-protein kinase (membrane translocator protein)	1677/33568_R	2576-4196	NP_828679	2.64*	0.0002
Carbohydrate metabolism and glycolysis	*glpF*	Glycerol uptake facilitator	266/038-2	1960-2725	NP_786656	2.56*	0.0002
	pgm	Phosphoglycerate mutase	571/14586[e] 1677/33568_R[e]	5028-5244 21-227	NP_784032	2.87*	0.0001
	ydaM	Glycosyl transferase family 2	198/011	15258-15439	NP_267421	3.63*	<0.0001
Nucleotide metabolism	*adD*	Adenosine deaminase	241/027-2[e] 1693/4194_R[e]	4975-5018 23-835	NP_965445	6.12*	<0.0001
	cdc8(*cmk*)	Thymidylate/cytidylate kinase	392/13722-13825	660-1238	NP_955193	2.51	<0.0015
	fhs	Formyltetrahydrofolate synthetase	1162/13850-13931_R	1396-2994	NP_785345	2.89*	<0.0001
	guaC	GMP reductase	1021/033-2_R[e] 2.256/033-1[e]	7225-7856 24-140	P60565	8.52*	<0.0001
	hpt	Hypoxanthine-guanine phosphoribosyltransferase	1494/18518_R[e]	716-1207	NP_964541	2.65*	<0.0001
	nrdD	Anaerobic ribonucleoside-triphosphate reductase	1376/15334_R[e]	24-653	ZP_00046952	2.83*	<0.0001
		Class III, large subunit	1225/13554_R[e]	35-1401			
	ptd	Purine transdeoxyribosylase (nucleoside deoxyribosyltransferase I)	1693/4194_R	893-1374	AAL73113	2.91*	<0.0001
	purC	Phosphoribosylaminoimidazole-succinocarboxamide synthase	1026/036-1_R	297-977	ZP_00286446	5.75*	<0.0001
	purK	Phosphoribosylaminoimidazole carboxylase II	441/13345	24-967	NP_471220	2.76*	<0.0001
	purS	Phosphoribosylformylglycinamidine synthase, PurS component	1026/036-1_R	977-1194	AAD12624	5.67*	<0.0001
	pbuX	Xanthine permease	1079/054-5_R	1249-1867	ZP_00046972	3.06*	<0.0001
	pbuG	Hypoxanthine/guanine permease	1522/20655_R	1-562	ZP_00047460	20.14*	<0.0001
	pyrD	Dihydroorotate dehydrogenase, catalytic subunit	984/018-3_R	23-203	ZP_00045882	2.90	0.0078
	rtpR	Ribonucleotide-triphosphate reductase, α-subunit	1014/031-1_R	23-2047	ZP_00046381	2.55*	<0.0001
	xpt	Xanthine/adenine/guanine phosphoribosyltransferase	1079/054-5_R	638-1180	ZP_00046973	3.04*	<0.0001
Binding and transport	*ABC6 MC1* (*glnP*)	ABC-type amino acid transporter His/Glu/Gln/Arg family, permease component	1224/13552_R	74-677	NP_965164	2.62	0.0385
	ABC6ATP6 (*glnQ*)	Amino acid ABC transporter, ATP-binding protein	1224/13552_R	746-1333	NP_965163	2.68	0.0123
	ABC7	ABC transporter, ATPase and permease components	1283/13928_R	24-698	ZP_00045914	2.86	0.0005
Phage proteins	*hyp410-1*	Phage portal protein	410/14512-14063	35-1278	ZP_00046441	3.23*	0.0003
	hyp456-2	Phage protein	456/13492	203-416	NP_607392	2.90	0.0020
	hyp551-1	Phage protein	551/14238	27-785	ZP_00046442	3.15*	0.0004
	hyp578-1	Phage protein	578/14690	30-412	NP_930700	2.73*	<0.0001
	hyp705-3	Phage protein	705/18213	1554-1741	ZP_00046427	2.21	0.0009
	hyp705-6	Phage terminase, small subunit	705/18213	4434-4929	NP_785880	3.80*	0.0002
	hyp705-7	Phage terminase, large subunit	705/18213	4929-5694	ZP_00046440	3.20*	0.0001
	hyp1177-1	Phage scaffolding protein	1177/14643-14700	667-1146	ZP_00046443	2.59	0.0005
	hyp1177-2	Main capsid phage protein	1177/14643-14700_R	1200-2273	ZP_00046444	2.60	0.0021
	hyp1177-4	Phage protein	1177/14643-14700_R	2666-2994	ZP_00046446	2.44	0.0014
	hyp1177-5	Phage protein	1177/14643-14700_R	3040-3396	ZP_00061146	2.92*	0.0004
	hyp1177-6	Phage protein	1177/14643-14700_R	3444-3848	ZP_00046447	2.57	0.0024
	hyp1177-9	Phage core tail protein	1177/14643-14700_R	5514-5809	ZP_00046450	2.78	0.0011
	hyp1320-1	Phage protein	1320/14209_R	103-654	NP_945294	2.72	0.0025
	hyp1332-1	Phage-related minor tail protein	1332/14517_R	25-247	ZP_00046452	2.32	0.0026
	xkdK	Phage sheath tail protein	1177/14643-14700_R	4027-5465	ZP_00046449	2.58	0.0020
	yejH	Helicase	578/14690	1179-1560	CAC04160	3.69*	0.0001
	yqbP	Phage protein	1332/14517_R	247-700	ZP_00046453	2.30	0.0050
Miscellaneous	*apf1*	Aggregation-promoting factor	197/010-2	3962-4658	AAN78450	4.31*	<0.0001
	bclA	Collagen-like protein	1571/25128_R	21-518	ZP_00030693	2.90*	0.0001
	rep	Plasmid replication protein	1169/14151-14562_R	833-1015	NP_863615	2.37*	<0.0001

[a] Each tentatively assembled sequenced fragment (contig) of the *L. helveticus* CNRZ32 genome was assigned a unique sequence identification number (ID) during the manufacturing of the microarrays.

[b] Indicates the start of the first oligonucleotide probe and the end of the last probe within a given contig used in the calculation of the expression value for a particular ORF.

[c] Fold change in normalized microarray signal intensity during growth in milk versus MRS. Given are average values calculated from three independent repeats performed in duplicate. Asterisks indicate significance of the differences after applying Bonferroni's correction at an overall level of 0.1.

[d] *P* values were calculated by the mixed-model method.

[e] Fragments of the same gene were present on two different contigs; these fragments were combined for the analysis.

TABLE 6. Summary of the differences observed in expression of *L. helveticus* CNRZ32 genes during growth in milk and MRS medium

Gene function or type	Induced during growth in:	
	Milk	MRS[a]
Proteolysis	Casein utilization proteinases Oligopeptide transporters Aminopeptidases Endopeptidases Aminoacylase/carboxypeptidase	Stress-related proteases Di-/tripeptide transporters Dipeptidases Iminopeptidase Signal peptidase
Amino acid metabolism	Serine-P catabolism Cysteine metabolism	Asparagine/aspartate catabolism Lysine biosynthesis pathway
Carbohydrate metabolism	Lactose utilization Glycerol uptake	Glucose utilization
Lipases and esterases	Triacylglycerol lipase	Phosphoesterase Stress-related lipase/esterase
Nucleotide metabolism	Purine salvage and de novo synthesis	ND
Citrate utilization	Putative citrate transporter Citrate lyase regulator	Citrate lyase complex Fumarate reductase
Binding and transport	Glutamine ABC-type transporter	ABC-type transporters involved in [Fe-S] cluster assembly
Phage proteins	Phage-related proteins (18 in total)	ND
Miscellaneous	Collagen-binding protein Aggregation-promoting protein Plasmid replication protein Helicase	Stress-related proteins Competence protein Recombination protein

[a] ND, no overexpressed genes of this group were detected.

measured more substantive expression changes than did microarray analysis.

Growth experiments in chemically defined media. Initial trials indicated that the addition of serP to CDM at 2.75 mM, 5.5 mM, or 11 mM had no influence on growth of *L. helveticus* CNRZ32, as judged by 24-h yields (data not shown). However, additional experiments suggested that supplementation with cheese-derived phosphopeptides increased the specific growth rate of *L. helveticus* CNRZ32 (Table 7).

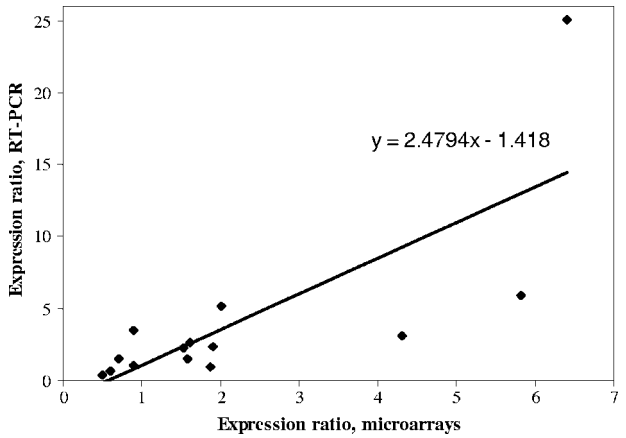

FIG. 1. Correlation of expression ratios from microarray profiling and RT-PCR. Total RNA was extracted from milk-grown and MRS-grown cultures of *Lactobacillus helveticus* CNRZ32 and served as a template for cDNA synthesis to be used in microarrays and RT-PCR experiments. The calculated expression ratios (*n*-fold changes) obtained from log-transformed data of 14 genes (Table 1) are shown for microarray experiments (horizontal axis) and RT-PCR (vertical axis). The best-fit linear regression curve is shown along with the calculated equation.

DISCUSSION

The growth of *L. helveticus* in milk involves a number of physiological activities, including lactose fermentation, casein hydrolysis, and amino acid metabolism, which are also known to influence flavor development in bacteria-ripened cheeses (12).

Carbohydrate and lipid metabolism. Since lactose is a major source of carbohydrate for the growth of lactobacilli in milk, the upregulation of genes for lactose permease (*lacS*) and β-galactosidase (*lacM*) was expected (11) and observed (Table

TABLE 7. Growth rates of *L. helveticus* CNRZ32 in a chemically defined medium supplemented with different compounds

Supplement	Growth rate (μ_{max})[a]		Generation time (min)	% Difference vs. control
	Mean	SD		
None (control)	0.129	0.007	323	0
Phosphopeptides, 1.5 mM[b]	0.167	0.016	248	23
Serine, 6 mM	0.144	0.020	290	10
Phosphoserine, 6 mM	0.148	0.022	280	13

[a] Values were obtained from three independent trials.
[b] Concentration of organic phosphate (28).

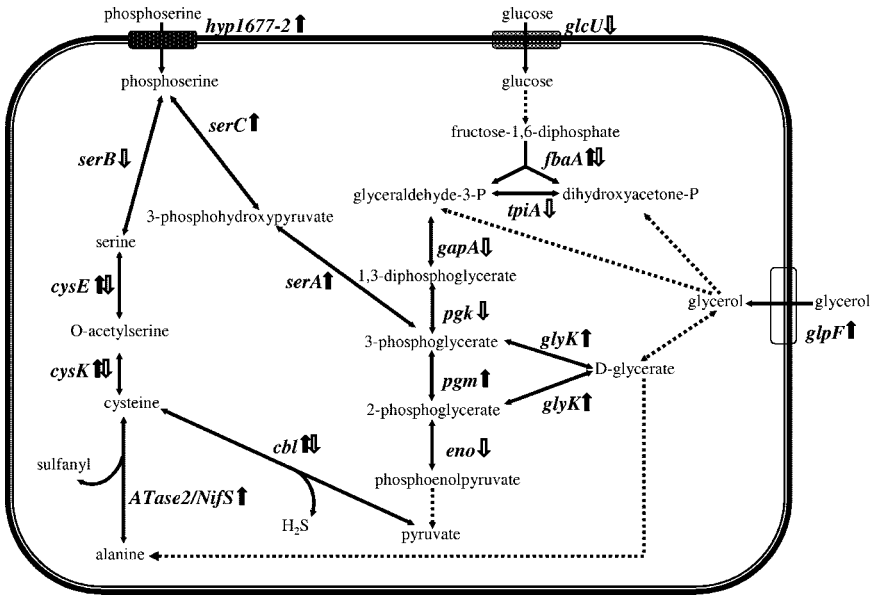

FIG. 2. Map of selected metabolic pathways in *Lactobacillus helveticus* CNRZ32. The different enzymatic steps are represented by the correspondent gene designations. Dotted arrows indicate several enzymatic steps or the absence of data regarding a particular gene. Arrows next to the gene symbols indicate either the upregulation (up arrow) or the downregulation (down arrow) of this gene during the growth in milk compared with growth in MRS; two opposite arrows indicate no change in the expression. *glcU*, gene encoding glucose/ribose uptake protein; *fbaA*, gene encoding fructose-1,6-biphosphate aldolase; *tpiA*, gene encoding triosephosphate isomerase; *gapA*, gene encoding glyceraldehydes 3-phosphate dehydrogenase; *pgk*, gene encoding phosphoglycerate kinase; *pgm*, gene encoding phosphoglyceromutase; *eno*, gene encoding enolase; *glpF*, gene encoding the glycerol uptake facilitator; *hyp1677-2*, gene encoding hypothetical membrane serine/threonine-protein kinase (membrane translocator); *serA*, gene encoding phosphoglycerate dehydrogenase; *serB*, gene encoding hypothetical phosphoserine phosphatase (*ycsE*); *cysE*, gene encoding serine *O*-acetyltransferase; *cysK*, gene encoding cysteine syntase; *ATase2/NifS*, gene encoding aminotransferase class V/cysteine desulfurase; *cbl*, gene encoding cystathionine β-lyase. Hypothetical transporter proteins are indicated by rectangles.

2). Similarly, the presence of 2% glucose in MRS medium likely explains the observed upregulation of genes for its correspondent transporter (*glcU*), glucose-induced repressor (*ccpA*), triosephosphate isomerase (*tpiA*), and central glycolytic gene regulator (*ygaP*/*cggR*). In *Bacillus subtilis*, the latter gene serves as a repressor for a downstream operon that includes genes encoding five enzymes of the glycolytic pathway: glyceraldehyde 3-phosphate dehydrogenase (*gapA*), phosphoglycerate kinase (*pgk*), triosephosphate isomerase (*tpi*), phosphoglycerate mutase (*pgm*), and enolase (*eno*) (7). This cluster has a similar structure (*ygaP*/*cggR-gapA-pgk-tpi*) in *L. helveticus* CNRZ32 and *Lactobacillus delbrueckii* subsp. *bulgaricus* (4), which suggests that YgaP/CggR also acts as a negative regulator for *gapA* expression in these organisms when no inducer is present. However, the microarray data shows small but significant upregulation of all genes of the cluster, *gapA* included, during the growth of *L. helveticus* CNRZ32 in MRS. An additional examination of the tiled microarray data also revealed a change in the expression of *pgk* under these conditions (R = 1.17; P = 0.0026). These results indicate that the entire *gapA* operon in *L. helveticus* CNRZ32 is derepressed in the presence of sugars, and similarly to *B. subtilis*, an mRNA processing event may be involved to provide for differential synthesis of the encoded proteins (7).

In *L. helveticus*, a putative *gapA* operon does not include *pgm* and *eno*. Instead, *pgm* is physically linked to the serine metabolism genes *serA* and *serC* and all three genes are strongly upregulated in milk (Fig. 2 and 3). The expression level of *fbaA*, which encodes fructose-1,6-biphosphate aldolase in MRS-grown cells, remained unchanged (R = 1.03; P = 0.5550). Unchanged or decreased expression of genes encoding the enzymes of the earlier steps of glycolysis (i.e., *fbaA*, *gapA*, and *pgk*) in the milk-grown culture suggests that the upregulation of *pgm* may be triggered by the concentration of its substrate, 3-phosphoglycerate. This may be due to the increased expression of both *serA* (encodes 3-phosphoglycerate dehydrogenase) and the glycerate kinase gene *glyK* (Fig. 2). Enolase catalyzes the next enzyme of glycolysis (Fig. 2), and

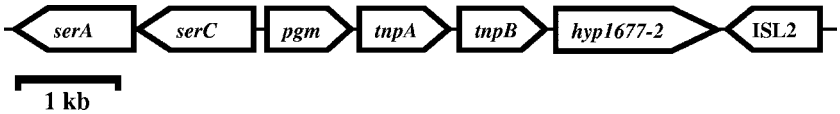

FIG. 3. Schematic representation of a locus containing genes of serine metabolism in *Lactobacillus helveticus* CNRZ32. Abbreviations: *serA*, gene encoding phosphoglycerate dehydrogenase; *serC*, gene encoding phosphoserine aminotransferase; *pgm*, gene encoding phosphoglycerate mutase; *tnpA*, gene encoding transposase; *tnpB*, gene encoding transposase; *hyp1677-2*, gene encoding hypothetical membrane serine/threonine-protein kinase (membrane translocator); ISL2, insertion sequence.

the correspondent gene, *eno*, was downregulated in the milk-grown culture ($R = 0.88$; $P = 0.0088$). If we assume the presence of specificity of GlyK to 2-phosphoglycerate as well, the upregulation of *glyK* suggests that the theoretically increased pool of 2-phosphoglycerate may be converted into D-glycerate that is further utilized in biosynthetic processes, e.g., L-alanine production (Fig. 2). Alternatively, flux through residual enolase activity is also possible. The tiled microarrays also identified the induction of the gene *glpF* for a predicted glycerol uptake facilitator protein of the aquaporin family which provides for energy-independent reversible diffusion of glycerol (27). Milk is not an apparent source of glycerol, but it may be a product of milk triacylglycerol hydrolysis by lipase(s). Coincidently, the microarray data revealed the upregulation of a *lip*(*con hyp069A1*) gene which may encode triacylglycerol lipase in milk-grown culture. Simultaneous upregulation of both *glpF* and *lip* may be considered an indication that *L. helveticus* CNRZ32 is able to utilize milk glycerol-containing lipids. The potential pathways for the further conversion of glycerol, such as to D-glycerate via D-glyceraldehyde, should be subjected to further investigation.

Proteolysis. Since *L. helveticus* CNRZ32 has multiple amino acid auxotrophies (6), the rapid growth of this bacterium in milk requires several enzymes to procure essential amino acids from casein. These include extracellular, cell envelope-associated proteinases to hydrolyze caseins; transport systems to take up liberated oligopeptides and free amino acids; and intracellular peptidases to further degrade oligopeptides into smaller peptides (endopeptidases) and free amino acids (aminopeptidases) (5). As expected, data from annotated arrays that showed growth in milk raised the expression level of genes for one previously described cell envelope-associated serine proteinase, PrtH (32), and for an additional hypothetical PrtH2 protease as well as a gene for the PrtM protein that is presumably required for proteinase maturation. Tiled arrays permitted the identification of two novel cell wall-associated serine proteinases (PrtH3 and PrtH5) that were also upregulated in milk-grown culture. The presence and expression of several cell wall proteinases in *L. helveticus* CNRZ32 may possibly reflect their different specificities toward α- and β-caseins as previously suggested for another *L. helveticus* strain (16). Milk-grown cells also showed significantly higher expression levels of genes for oligopeptide transport as well as a variety of intracellular peptidases. One of the peptidase genes induced in milk, *pepO2*, encodes an endopeptidase with postprolyl specificity that contributes to the ability of *L. helveticus* CNRZ32 to hydrolyze bitter peptides in cheese (37). The array data also confirm previous results regarding the importance of the peptidases PepN, PepX, PepV, PepR, and PepT for adequate growth of *L. helveticus* in milk (6).

In contrast, the upregulation of genes for components of the Dtp system for di-/tripeptide transport and peptidases PepI, PepQ, and PepD during the growth of *L. helveticus* CNRZ32 in MRS likely reflects an abundance of small, proline-rich peptides in this medium. Growth in MRS also led to the upregulation of genes for several stress-related and housekeeping proteases.

Amino acid metabolism. The conversion of amino acids into volatile and nonvolatile compounds by LAB in cheese is thought to represent the rate-limiting step in the development of mature flavor and aroma (8, 46, 50). However, the growth of *L. helveticus* CNRZ32 in milk had little effect on the expression of genes for amino acid biosynthesis, presumably due to the ready supply of amino acids in this medium. An exception was the *ATase2*/*nifS2* gene, whose product may possess dual activity and function as a cysteine desulfurase (EC 2.8.1.7), which catalyzes the hydrolysis of cysteine to alanine, and as a class V aminotransferase, which may participate in the catabolism of Leu and Val.

Draft sequencing data and array results from milk-grown *L. helveticus* CNRZ32 identified a hypothetical pathway for serP utilization in *L. helveticus* that involves the uptake of serP as a free amino acid or, more likely, within phosphorylated peptides (Fig. 2). Once inside the cell, free serP could be produced by the action of peptidase(s), followed by conversion to serine by phosphoserine phosphatase (SerB; EC 3.1.3.3) or transaminated by phosphoserine aminotransferase (SerC; EC 2.6.1.52) to 3-phosphohydroxypyruvate. The latter can be converted through the action of 3-phosphoglycerate dehydrogenase (SerA; EC 1.1.1.95) into a compound that feeds into the central glycolytic pathway, 3-phosphoglycerate, which may then be converted to 2-phosphoglycerate by phosphoglycerate mutase (Pgm; EC 5.4.2.1) (Fig. 2).

As shown in Fig. 3, *serA*, *serC*, and *pgm* are located in close proximity with each other, and microarray data showed that all three genes are significantly upregulated during the growth of *L. helveticus* in milk. Additionally, the sequence and microarray data indicated the presence of another upregulated ORF located further downstream of *pgm*. This ORF, named *hyp1677-2*, could encode a protein similar to a number of membrane-associated serine/threonine protein kinases and membrane translocase proteins. In contrast, the expression of the most likely ortholog for phosphoserine phosphatase (*serB*), designated *serB*/*ycsE*, was not changed during the growth of *L. helveticus* CNRZ32 in milk ($R = 1.05$; $P = 0.2571$). The upregulation of *serA*, *serC*, and *pgm* in milk-grown cultures of *L. helveticus* CNRZ32, and an unchanged level of *serB*/*ycsE* suggest that this strain may actively catabolize phosphoserine (Fig. 2). Previous studies have suggested that serine, but not serP, is metabolized (deaminated) by the strains *Lactobacillus casei* and *Lactobacillus plantarum* (23, 26). Based on these data, Liu et al. (26) speculated that serP cannot be metabolized unless it is converted to serine by action of phosphatase. However, the data presented here indicate that in *L. helveticus*, the metabolism of serP may also proceed via an alternative pathway which does not involve consecutive dephosphorylation and deamination. Preliminary experiments suggest that a phosphopeptide fraction isolated from 10-month-old Herrgard cheese enhanced the growth of *L. helveticus* CNRZ32 in CDM. This fraction contains short (<10 amino acid residues) phosphopeptides (28) which should be readily transported by the Opp system and serve as substrates for intracellular peptidase(s). However, the additional investigation is required to establish whether the observed growth stimulation property of phosphopeptides is due to the presence of serP residues.

Citrate metabolism. Citrate may serve as an additional source of metabolic energy for LAB in milk (13). Citrate catabolism is also associated with the production of important cheese flavor compounds, such as succinate, diacetyl, acetaldehyde, and acetoin (12, 17).

An analysis of the CNRZ32 genome established that genes for citrate metabolism are organized in an operon-like structure similar to those found in other LAB, such as *Lactococcus lactis* (31) and *Leuconostoc* spp. (29). The citrate operon in *L. helveticus* CNRZ32 includes five ORFs, *citRCDEF*, which are predicted to encode a transcriptional regulator, citrate lyase ligase, and γ-, β-, and α-subunits of citrate lyase, respectively. Unlike the citrate operons of *Lactococcus lactis* and *Leuconostoc* spp., however, the *L. helveticus* CNRZ32 cluster is preceded by a codirectional ORF (*yflS*) predicted to encode a C_4-dicarboxylate transporter with 31% identity over 495 amino acids to an experimentally characterized citrate carrier protein, CitT, from *Escherichia coli* (34). While *yflS*/*citT* was upregulated during the growth of *L. helveticus* CNRZ32 in milk, all genes of the *citRCDEF* cluster except *citR* were induced in MRS-grown culture. In other LAB, citrate operon transcription is regulated by a transcriptional activator, CitI, whose gene is located immediately upstream of *citC*, and is induced by citrate and acidic conditions (30, 31). The *citI* gene in *L. helveticus* CNRZ32 is found at a distal locus and, like *yflS*/*citT* but unlike *citCDEF*, was upregulated in milk-grown culture. The pH values of the milk- and MRS-grown cultures of *L. helveticus* CNRZ32 in our experiments were similar, but because citrate concentrations were not measured in milk used in the present study, it is not clear whether the expression of citrate metabolism genes in this organism may have been affected by differences in medium citrate concentrations.

In *Lactococcus lactis* and *Leuconostoc* spp., two other genes, *citG* and *citX*, which encode enzymes required for the activation of citrate lyase, are adjacent to and cotranscribed with the genes for citrate lyase ligase and citrate lyase (30, 31). However, in *L. helveticus* CNRZ32, these genes are found in separate loci and are expressed in a dissimilar manner; *citG* is upregulated in milk-grown cells, and *citX* is not differentially expressed (data not shown). This suggests that the situation with *L. helveticus* CNRZ32 may be analogous to that with *Klebsiella pneumonia* in that it employs distinct regulatory mechanisms for *citG* and *citX* (36).

Growth in MRS also induced the expression of genes that participate in the conversion of oxaloacetate to succinate: *aspC* (aspartate aminotransferase) and two of at least three paralogs of *frdC* (fumarate reductase). At the same time, no upregulation was recorded for *gabD*, which encodes succinic semialdehyde dehydrogenase (EC 1.2.1.16), an enzyme that further converts succinate to succinate semialdehyde. Taken together, these data suggest that *L. helveticus* has a functional pathway of citrate metabolism, possibly leading to succinate production. Indeed, a recent report by Torino et al. (42) demonstrates that *L. helveticus* can metabolize citrate into succinate, an important cheese flavor compound.

Nucleotide metabolism. Milk is a poor source of purine bases available for utilization by microorganisms (15). Microarray data are in good agreement with reports which have shown that purine biosynthesis is required for the growth of LAB in milk (21). As expected, the growth of *L. helveticus* CNRZ32 in milk led to the induction of two operons, *purCQLFMNHD* and *purAB*, whose products are required for the conversion of 5′-phosphoribosyl-1-pyrophosphate to inosine-5′-phosphate (IMP), a central intermediate in purine anabolism (21). An important cofactor in the IMP biosynthesis, 10-formyl-tetrahydrofolate, is produced from formate and tetrahydrofolate by formate-tetrahydrofolate synthase whose correspondent gene, *fhs*, was also upregulated in milk-grown culture. Genes that encode enzymes for further conversion of IMP to AMP (*purA* and *purB*), XMP (*guaB*), or GMP (*guaA*) were also upregulated in milk-grown cells.

The tiled microarrays also showed strong upregulation of genes encoding xanthine and hypoxanthine/guanine transporters (*pbuX* and *pbuG*, respectively) as well as *xpt* and *hpt*, whose products catalyze the conversion of these compounds into GMP, XMP, and IMP. Two additional purines that salvage/interconversion genes, *adD* (adenosine deaminase) and *ptd* (purine transdeoxyribonuclease) (19), were also strongly induced in the milk culture. These data suggest *L. helveticus* CNRZ32 relies on both salvage and de novo synthesis pathways to obtain purine nucleotides during the growth in milk.

In contrast, the expression levels of genes for pyrimidine biosynthesis were not significantly different in milk- and MRS-grown cultures. An exception was *pyrD*, which was overexpressed in milk-grown cultures. It seems to reflect a direct influx of orotate present in milk (40). The tiled microarrays, however, identified three other genes that were overexpressed in the milk-grown cultures, *cdc8*/*cmk*, *rtpR*, and *nrdD*, that were predicted to encode thymidylate/cytidylate kinase; ribonucleotide-triphosphate reductase, class II (3); and ribonucleoside-diphosphate reductase, class III (43), respectively, enzymes for the pyrimidine salvage pathway. These results suggest that *L. helveticus* CNRZ32 may rely more on rescuing existing pyrimidine compounds than on de novo synthesis during the growth in milk.

Phage genes. During cheese ripening, the lysis of cheese bacteria, such as *L. helveticus*, is considered to be a beneficial process because it facilitates the release of intracellular peptidases which then participate in proteolysis (1). Cells of *L. helveticus* are known to undergo extensive lysis during the early stages of Swiss cheese molding and ripening (45), and prophage activation may explain this observation (25). Microarray results from this study showed that the growth of *L. helveticus* CNRZ32 in milk was accompanied by the upregulation of a large number of phage-related genes (Table 5). Since genes for phage lysin were not identified in this group, however, further work is needed to confirm a role for prophage induction in the autolysis of *L. helveticus* CNRZ32.

ACKNOWLEDGMENTS

This study was supported by Dairy Management, Inc., through the Wisconsin Center for Dairy Research and the Western Dairy Center, and by Chr. Hansen, Inc.

The help of Brian Yandell, Tom Tabone, and Mateo Budinich, University of Wisconsin—Madison, and Michael Hogan, NimbleGen, Inc., with statistical analysis is highly appreciated.

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